UTILITY PATENT APPLICATION TRANSMITTAL

(Only for nonprovisional applications under 37 CFR 1.53(b))

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First Named Inventor or Application Identifier

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"EXPRESS MAIL CERTIFICATE"

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APPLICATION ELEMENTS *See MPEP chapter 600 concerning utility patent application contents.*	
1. ☒ The Commissioner is hereby authorized to charge indicated fees and credit any overpayments to Deposit Account No. 19-2570 ☒ General Authorization to charge any and all fees under 37 CFR 1.16 or 1.17, including petitions for extensions of time, relating to this application. *(37 CFR 1.136(a)(3))* *(Submit an original, and a duplicate for fee processing)*	7. ☒ The Title of the Invention: FLUORESCENCE ENERGY TRANSFER BY COMPETITIVE HYBRIDIZATION
2. ☒ The total fee is calculated as shown below: Basic Filing fee $790.00 Total Claims 13 - 20 = 0 x $22 $ 0.00 Independent Claims 2 -3 = 0 x $82 $ 0.00 ☐ Multiple Dependent Claim present. $270 TOTAL FILING FEE $790.00 ☐ Cancel in this application original claims _to __of the prior application before calculating the filing fee. Charge $790.00 to the above indicated Deposit Account.	8. ☐ Nucleotide and/or Amino Acid Sequence Submission a. ☐ Computer Readable Copy b. ☐ Paper Copy (identical to computer copy) c. ☐ Statement verifying identity of above copies d. ☐ Use the identical computer-readable form filed in Application No. _____, filed ___ _ as the computer-readable form for the instant application. (37 CFR 1.821(e)) **ACCOMPANYING APPLICATION PARTS**
3. ☒ Specification excluding Drawings [*Total Pages*] 14	9. ☐ Information Disclosure Statement (IDS) a. ☐ PTO-1449 b. ☐ Copies of all IDS Citations
4. ☒ Drawing(s) *(35 USC 113)* [*Total Sheets*] 1	10. ☐ Assignment Papers (cover sheet & document(s))
5. ☒ Declaration and Power of Attorney [*Total Pages*] 3 a. ☐ Newly executed (original or copy) b. ☐ Copy from a prior application (37 CFR 1.63(d)) *(for continuation/divisional with Box 17a completed)* c. ☒ Unsigned Declaration *[Note Box 6 below]* i. ☐ DELETION OF INVENTOR(S) Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b).	11. ☐ Prior Application is Assigned to: _____ *(for continuation/divisional with Box 17a completed* 12 ☐ Preliminary Amendment [*Total Pages*] 13 ☒ Return Receipt Postcard (MPEP 503) *(Should be specifically itemized)* 14. ☐ Certified Copy of Priority Document(s) *(if foreign priority is claimed)* 15. ☐ Transfer all references cited by Applicants or by the Examiner from the parent Application Serial No. _ _____ filed_____. ☐ PTO - 1449 Form 16. ☐ Other: _____
6. ☐ Incorporation By Reference *(useable if Box 5b is checked)* The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 5b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.	

17. Priority Information, *check appropriate box and supply the requisite information*

a. The accompanying application is a ☐ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application No: 0 filed 0.

b ☒ Benefit is claimed under Title 35, United States Code, Section 119(e) of the following Provisional Applications:
Application No. 60/039,583 filed February 28, 1997

c. ☐ Please amend the specification by inserting before the first line the sentence:
This is a continuation/divisional of application Serial No. _____ filed _____.

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Fluorescence Energy Transfer by Competitive Hybridization

Scope of the Invention

This invention relates to unequal length complementary probes which have a fluorophore on one probe and a quencher on the other. The fluorophore and quencher are juxtaposed in a manner such that the proximity of the quencher to the fluorophore quenches the fluorescence of the fluorphore. These probes are useful in detecting nucleotides with sequence complemenarity. The detection capabilities reside in the competitive hybridization resulting from the use of unequal length probes, and the subsequent decrease in quenching brought about by this competitive hybridization.

Area of the Invention

Nucleic acid amplification techniques have added to the collection of techniques by which very small quantities of a nucleotides can be enhanced to a concentration where they can be detected by some means. Several amplification techniques have become available. The most wide-spread technique is that of polymerase chain reaction, or PCR as it is now commonly called. While the amplification techniques increase the number of target nucleotide sequences available for detection, or recovery and use, a sensitive method is needed to detect the amplification product. Also, amplification technologies benefit from real-time monitoring of the amplification process. Real-time monitoring can detect non-reactive amplification runs, or detect inefficiencies in the process. Quantification of oligonucleotide burden may also be possible with real-time monitoring if such monitoring can be done without interfering with the amplification reaction.

The procedure of this invention is based on the fluorescence energy transfer between a fluorophore labeled probe and a quencher labeled probe, with sequence complementarity to each other. The probes used are of unequal length favoring the annealing of one probe to the target nucleic acid sequence over annealing to its complementary probe. In the absence of nucleic acids with sequences complementary or identical to the probe sequences (target sequence), the two probes would anneal to each other. When the two probes are annealed to each other, the proximity of the quencher to the fluorophore produces quenching of the fluorescence of the fluorophore. In the presence of nucleic acid with sequences complementary or identical to the probe sequence, some of the fluorophore labeled probe will hybridize to the nucleic acid with the complementary sequence and be separated from the quencher and yield increased (unquenched) fluorescence. This difference in fluorescence can be used for specific detection of the presence of nucleic acids with the target sequences.

Summary of the Invention

In a first aspect, this invention relates to a method for monitoring nucleic acid amplification comprising performing nucleic acid amplification on a target polynucleotide wherein the amplification is carried out by any method using a first primer and second primer of varying in length by about at least 3 base pairs and capable of hybridizing to said target polynucleotide and a first oligonucleotide probe and a second shorter oligonucleotide probe varying in length by about at least 2 base pairs; the first probe having a fluorophore; the second being complementary with the first probe and having a quencher molecule capable of quenching the fluorescence of said fluorophore, the fluorophore and quencher being attached on their respective probes at positions which results in the quencher molecule quenching the fluorescence of the fluorophore when the probes are hybridized, wherein the longer probe binds preferentially to the target polynucleotide and when preferentially bound to the target polynucleotide the fluorescence intensity of the fluorophore is greater than the fluorescence intensity of the fluorophore when hybridized to the second probe, and monitoring the fluorescence of the fluorophore, the generation of fluorescence corresponding to the occurrence of nucleic acid amplification.

It also relates to a method for detecting the presence of a target polynucleotide using the unequal length probes.

The annealed probes with fluorophore and quencher are also part of this invention.

Brief Description of the Drawings

Fig 1 illustrates the fluorescence signal obtained from a PCR amplification of target HCV RNA using unequal length probes where the longer probe had fluorophore on the 5' terminal carbon, the shorter probe had a quencher on the 3' terminal carbon and was prepared by deleting three base pairs from the 5' terminus of the longer probe.

Detailed Description of the Invention

This invention is used in conjunction with the amplification of a target polynucleotide by by any method. These amplification techniques include PCR, ligase chain reaction (LCR), gap LCR, transcription mediated amplification (TAM), nucleic acid sequence based amplification (NASBA), and strand displacement amplification (SDA).

PCR is of greatest interest. PCR is described in many references, such as Innis et at, editors, PCR Protocols (Academic Press, New York, 1989); Sambrook et at, Molecular Cloning, Second Edition (Cold Spring Harbor Laboratory, New York, 1989);

and the like. The binding site of the oligonucleotide probe is located between the PCR primers used to amplify the target polynucleotide. The PCR may be carried out using any polymerase. Because the intensity of the fluorescence signal intensifies as more replicates are made of the target polynucleotide, any polymerase which increased the number of target polynucleotides will work in this method. Preferably, PCR is carried out using a thermostable polymerase. The preferred enzyme is a Taq DNA polymerase, e.g. Amplitaq™. (Perkin-Elmer, Norwalk, Conn.), or an equivalent thermostable DNA polymerase. The annealing temperature of the PCR will be about 5 degree - 10 degree C. below the melting temperature of the oligonucleotide probes employed. The polymerase Pwo has also been use with success in this invention.

The term "oligonucleotide" as used herein includes linear oligomers of natural or modified monomers or linkages, including deoxyribonucleosides, ribonucleosides, and the like, capable of specifically binding to a target polynucleotide by way of a regular pattern of monomer-to-monomer interactions, such as Watson-Crick type of base pairing, or the like. Usually monomers are linked by phosphodiester bonds or analogs thereof to form oligonucleotides ranging in size from a few monomeric units, e.g. 3-4, to several tens of monomeric units. Whenever an oligonucleotide is represented by a sequence of letters, such as "ATGCCTG," it will be understood that the nucleotides are in 5' fwdarw 3' order from left to right and that "A" denotes deoxyadenosine, "C" denotes deoxycytidine, "G" denotes deoxyguanosine, and "T" denotes thymidine, unless otherwise noted. Analogs of phosphodiester linkages include phosphorothioate, phosphorodithioate, phosphoranilidate, phosphoramidate, and the like. Generally, oligonucleotide probes of the invention will have a sufficient number of phosphodiester linkages adjacent to its 5' end so that the 5' fwdarw 3' exonuclease activity employed can efficiently degrade the bound probe to separate the reporter and quencher molecules.

"Perfectly matched" in reference to a duplex means that the poly- or oligonucleotide strands making up the duplex form a double stranded structure with one other such that every nucleotide ineach strand undergoes Watson-Crick base pairing with a nucleotide in the other strand. The term also comprehends the pairing of nucleoside analogs, such as deoxyinosine, nucleosides with 2-aminopurine bases, and the like, that may be employed. Conversely, a "mismatch" in a duplex between a target polynucleotide and an oligonucleotide probe or primer means that a pair of nucleotides in the duplex fails to undergo Watson-Crick bonding.

Oligonucleotide probes of the invention can be synthesized by a number of approaches, e.g. Ozaki et at, Nucleic Acids Research, 20:5205-5214 (1992); Agrawal et at, Nucleic Acids Research, 18:5419-5423 (1990); or the like. The oligonucleotide probes of the invention are conveniently synthesized on an automated DNA synthesizer, e.g. an Applied Biosystems, Inc. Foster City, Calif.) model 392 or 394

DNA/RNA Synthesizer, using standard chemistries, such as phosphoramidite chemistry, e.g. disclosed in the following references: Beaucage and Iyer, Tetrahedron, 48:2223-2311 (1992); Molko et al, U.S. Pat. Nos. 4,980,460; Koster et al, U.S. Pat. No. 4,725,677; Caruthers et al, U.S. Pat. Nos. 4,415,732; 4,458,066; and 4,973,679; and the like. Alternative chemistries, e.g. resulting in non-natural backbone groups, such as phosphorothioate, phosphoramidate, and the like, may also be employed provided that the hybridization efficiencies of the resulting oligonucleotides and/or cleavage efficiency of the exonuclease employed are not adversely affected. Preferably, theoligonucleotide probe is in the range of 15-60 nucleotides in length. More preferably, the oligonucleotide probe is in the range of 18-30 nucleotides in length. The precise sequence and length of an oligonucleotide probe of the invention depends in part on the nature of the target polynucleotide to which it binds. The binding location and length may be varied to achieve appropriate annealing and melting properties for a particular embodiment. Preferably, the 3' terminal nucleotide of the oligonucleotide probe is blocked or rendered incapable of extension by a nucleic acid polymerase. Such blocking is conveniently carried out by the attachment of a reporter or quencher molecule to the terminal 3' carbon of the oligonucleotide probe by a linking moiety. Preferably, reporter molecules are fluorescent organic dyesderivatized for attachment to the terminal 3' carbon or terminal 5' carbon of the probe via a linking moiety. Preferably, quencher molecules are also organic dyes, which may or may not be fluorescent, depending on the embodiment of the invention. For example, in a preferred embodiment of the invention, the quencher molecule is fluorescent. Generally, whether the quencher molecule is fluorescent or simply releases the transferred energy from the reporter by non-radiative decay, the absorption band of the quencher should substantially overlap the fluorescent emission band of the reporter molecule. Non-fluorescent quencher molecules that absorb energy from excited reporter molecules, but which do not release the energy radiatively, are referred to herein as chromogenic molecules. There is a great deal of practical guidance available in the literature for selecting appropriate reporter-quencher pairs for particular probes, as exemplified by the following references: Clegg "Fluorescence resonance energy transfer and nucleic acids," Methods of Enzymology, 211: 353-389 (1992), Wu et al; "Resonance energy transfer: methods and applications," Anal. Biochem. 218: 1-13 (1994).; Pesce et at, editors, Fluorescence Spectroscopy (Marcel Dekker, New York, 1971); White et at, Fluorescence Analysis: A Practical Approach (Marcel Dekker, New York, 1970); and the like. The literature also includes references providing exhaustive lists of fluorescent and chromogenic molecules and their relevant optical properties for choosing reporter-quencher pairs, e.g. Berlman, Handbook of Fluorescence Sprectra of Aromatic Molecules, 2nd Edition (Academic Press, New York, 1971); Griffiths, Colour and Consitution of Organic Molecules (Academic Press, New York, 1976);

Bishop, editor, Indicators (Pergamon Press, Oxford, 1972); Haugland, Handbook of Fluorescent Probes and Research Chemicals (Molecular Probes, Eugene, 1992); Pringsheim, Fluorescence and Phosphorescence (Interscience Publishers, New York, 1949); and the like. Further, there is extensive guidance in the literature for derivatizing reporter and quencher molecules for covalent attachment via common reactive groups that can be added to an oligonucleotide, as exemplified by the following references: Haugland (cited above); Ullman et al, U.S. Pat. No. 3,996,345; Khanna et al, U.S. Pat. No. 4,351,760; and the like.

Exemplary reporter-quencher pairs may be selected from xanthene dyes, including fluoresceins, and rhodamine dyes. Many suitable forms of these compounds are widely available commercially with substituents on their phenyl moieties which can be used as the site for bonding or as the bonding functionality for attachment to an oligonucleotide. Another group of fluorescent compounds are the naphthylamines, having an amino group in the alpha or beta position. Included among such naphthylamino compounds are 1-dimethylaminonaphthyl-5-sulfonate, 1-anilino-8-naphthalene sulfonate and 2-p-touidinyl-6-naphthalene sulfonate. Other dyes include 3-phenyl-7-isocyanatocoumarin, acridines, such as 9-isothiocyanatoacridine and acridine orange; N-(p-(2- benzoxazolyl)phenyl)maleimide; benzoxadiazoles, stilbenes, pyrenes, and the like. Preferably, reporter and quencher molecules are selected from fluorescein and rhodamine dyes. These dyes and appropriate linking methodologies for attachment to oligonucleotides are described in many references, e.g. Khanna et al (cited above); Marshall, Histochemical J., 7:299-303 (1975); Mechnen et at, U.S. Pat. No. 5,188,934; Menchen et al, European pat. No. application 87310256.0; and Bergot et al, International application PCT/US90/05565. The latter four documents are hereby incorporated by reference.

There are many linking moieties and methodologies for attaching reporter or quencher molecules to the 5' or 3' termini of oligonucleotides, as exemplified by the following references: Eckstein, editor, Oligonucleotides and Analogues: A Practical Approach (IRL Press, Oxford, 1991); Zuckerman et al, Nucleic Acids Research, 15: 5305-5321 (1987)(3' thiol group on oligonucleotide); Sharma et al, Nucleic Acids Research, 19:3019 (1991)(3' sulfhydryl); Giusti et al, PCR Methods and Applications, 2:223-227 (1993) and Fung et al, U.S. Pat. No. 4,757,141 (5' phosphoamino group via Aminolink™. II available from Applied Biosystems, Foster City, Calif.); Stabinsky, U.S. Pat. No. 4,739,044 (3' aminoalkylphosphoryl group); Agrawal et al, Tetrahedron Letters, 31:1543-1546 (1990)(attachment via phosphoramidate linkages); Sproat et al, Nucleic Acids Research, 15:4837 (1987)(5' mercapto group); Nelson et al, Nucleic Acids Research, 17:7187-7194 (1989)(3' amino group); and the like. Preferably, commercially available linking moieties are employed that can be attached to an

oligonucleotide during synthesis, e.g. available from Clontech Laboratories (Palo Alto, Calif.).

Rhodamine and fluorescein dyes are also conveniently attached to the 5' hydroxyl of an oligonucleotide at the conclusion of solid phase synthesis by way of dyes derivatized with a phosphoramidite moiety, e.g. Woo et al, U.S. Pat. No. 5,231,191; and Hobbs, Jr. U.S. Pat. No. 4,997,928.

The selection of primers and probes used in this invention is at the choice of the practitioner. This invention places no unique requirements or restrictions on primer or probe choice. Such choices are within the skill of the art.

The probes can be of any length, so long as that length allows one to practice the invention. As a baseline the longer probe must have at least 3 base pairs; as a practical matter the longer probe will be comprised of more than 3 base pairs. However, there is no upper limit occasioned by the use of FETCH. The length of the longer probe is not dictated by the application of FETCH as it will work with any length of probe, over and above the basic requirement of being at least 3 base pairs long. As a practical matter the longer probe will have a length which insures that it hybridizes uniquely with the target polynucleotide and the shorter probe. The shorter probe will be at least 2 fewer base pairs than that of the longer probe. This is the basic standard for creating the shorter probe. It has been found that a good fit as regards the difference in length between the probes can be arrived at by calculating the dissociation temperature of the annealed probes. As a general rule the dissociation temperature of the primers needs to be higher than about 55 degrees C and lower than 90 degrees C. A convenient means for doing this calculation is to use the software called Gene Runer (Hastings Software, Inc.), for example version 3.04.

The shorter gene may be prepared as a 5' truncate of the longer gene. Or it may be a 3' truncate. A third option is to create the shorter probe by truncating both the 5' and the 3' end of the longer gene. Any one of these three forms of the shorter probe will work. While two or more truncated forms could be used, it is simplest to use just one form, preferably the 5' truncate form.

The fluorophore and the quencher can be located on any combination of base pairs so long as the fluorescence of the fluorophore is effectively quenched by the quencher when the two probes are hybridized. The simplest approach is to put the fluorophore on the 5' terminal nucleotide of the longer probe and the quencher on the 3' terminal nucleotide of the shorter probe. This approach can optimizes the quencher molecules affect on the fluorescence of the fluorophore. Preferably, fluorphore and quencher molecules are attached to the terminal 5' carbon and terminal 3' carbon of the probe by way of 5' and 3' linking moieties. However it has been demonstrated that, with at least a number of the fluorophores useful herein, that the fluorophore and the quencher can be situated remotely and still be operative. See for example U.S. patent

5,538,848 which discloses that a fluorophore and quencher may be separated by several nucleotides. That patent discloses work where a fluorophore and quencher are separated by at least 15 nucleotides, or more, and allegedly demonstrate utility. Likewise herein the fluorophore and quencher are permitted to be separated by numerous nucleotides, so long as that separation does not materially reduce the ability of the quencher to affect the signal of the fluorophore when the two probes are hybridized. Of course the fluorophore and/or quencher could be bonded to a nucleotide in the interior of the probes. But for reasons of ease of synthesis and for optimizing hybridization of probe to target and probe to probe, it is preferable to put the fluorophore and quencher on the 5' and 3' terminal ends of the probes, as noted above. In fact the fluorophore and quencher both be on the 5' or 3' terminal nucleotides of their respective oligonucleotides, and the assay will be functional. An alternative arrangement would be to put the fluorophore on the shorter probe and the quencher on the longer probe. Again in this arrangement the longer probe would preferentially bind to the target and thus be separated sufficiently from the probe with the fluorophore so as not to effectively quench its fluorescence. The preferred construct, however, is to have the fluorophore on the longer probe.

The following examples are given to illustrate the invention and are not intended nor should they be read to limit the scope of the invention as claimed in any fashion.

Examples

General Description

The use of fluorescence energy transfer by competitive hybridization (FETCH) was developed as an assay for the detection by PCR of hepatitis C virus (HCV) in human specimens. The forward probe, the longer probe, had a 6-FAM dye (carboxyfluoroscein) at the 5' position and a phosphate group at the 3' position (to prevent extension during PCR). The reverse probe, the shorter probe, had a TAMRA dye (N,N,N',N' tetramethyl-6-carboxyrhodamine) at the 3' position (no 3' OH thus no extension possible during PCR). FAM, a commonly used fluorescent dye, was used as the fluorophore. TAMRA, a fluorescent dye with absorption band overlapping the emission band of FAM, was used as the quencher in this application

Example 1

Selection of Primers and Probes

HCV polynucleotide sequences were identified from the literature. In order to have an assay which could catch all known HCV strains, two primers and two probes were selected which had polynucleotide sequences common to all reported strain RNA sequences. The primers were selected so that they would anneal to all the known HCV strains and possess satisfactory characteristics such as similar dissociation temperature, no extensive 3' complementarity to each other, etc. The ones chosen flank a 254 bp

segment of the 5' non-coding region of HCV from nucleotide number 3290 to 3543 as described in the literature. The HCV sequence data relied on, the primers, and the selected probes are identified in the following Tables I - II and IV (attached).

Table II

OLIGO	FORWARD PRIMER SEQUENCE	BASES	MW	LABEL
HCV	5'GCGTTAGTATGAGTGTCGTGCAGCCT	26	8008	None

OLIGO	REVERSE PRIMER SEQUENCE	BASES	MW	LABEL
HCVR2	5'GGTGCACGGTCTACGAGACC 3'	20	6124	None

Table III

TARGET SEQUENCE 3331-3350	
Probe C1	5'FAM CCGGGAGAGCCATAGTGGTC PO4
Probe C2	3'TAMRA GGCCCTCTCGGTATCAC

The unconventional presentation of the sequence of C2 (from 3' to 5') is to aid the visualization of its complementarity to C1.

Table IV

	BASES	MW	LABEL
Probe C1	20	6914	6-FAM
Probe C2	17	6065	TAMRA

Molecular weighs calculated by Gene Runer version 3.04 (Hastings Software, Inc.)

Example 2

Synthesis of Probes

Two probes were custom-synthesized at TriLink Biotechnologies, Inc. (11585 Sorrento Valley Rd., Suite 105, San Diego, CA 92121) They were prepared as follows:

Step 1. **Synthesis of Oligonucleotide**

The oligonucleotide was prepared on a support that will yield a 3' phosphate group upon deprotection (Glen Research Catalog No. 20-2913).

Step 2. **Addition of 6-FAM**

The support bound oligonucleotide was then reacted with 15 eqs. of 6-FAM amidite (Glen Research Catalog No. 10-5901) manually to ensure high efficiency.

Step 3. **Deprotection of Oligonucleotide**

The FAM labeled oligo was deprotected for 36 hours at room temperature with fresh conc. ammonium hydroxide. After deprotection the reagent was decanted, the beads rinsed, and the combined solutions dried.

Step 4. **Purification**

The FAM labeled oligonucleotide was purified using reverse phase HPLC. The FAM was only on full length material, and was a useful lipophilic handle allowing good separation. After purification, the compound was dried down in preparation for precipitation. The compound was precipitated from 0.3 M NaOAc using EtOH. The product was recovered by high speed centrifugation and washed twice with EtOH.

Step 5. Final Analysis

The dried product was resuspended in water, quantitated, and analyzed by HPLC for purity. The compound was then dried again in preparation for delivery.

Example 3

Detection of HCV RNA by Reverse Transcriptase-PCR Using FETCH

The assay was carried out as a one-step reverse transcription and polymerase chain reaction. HCV RNA was isolated and purified from human serum or plasma. The serum or plasma sample was lysed under highly denaturing conditions to inactivate RNAases and to insure isolation of intact RNA. The RNA was precipitated with ethanol and transferred to a QIAmp spin column (Qiagen, Chatsworth, CA) that binds RNA. The column was then washed and RNA eluted with water. The purified RNA template (10 µL) was mixed with HCV master mix (see Table V) (40 µL) and then reverse transcribed to DNA, amplified by PCR and detected in the same tube in the Perkin Elmer 7700 sequence analyzer as per Table VI. During PCR, some of the FAM labeled probe and some of the TAMRA labeled probe annealed to the PCR product thus reducing the quenching of FAM fluorescence and allowed increased fluorescence to be detected. The fluorescence of FAM increase with increasing number of cycles of thermocycling, corresponding with increases in amount of PCR product, as illustrated in Figure 1.

Table V

Typical HCV Master Mix		
Reagents	**1 Tube µL**	**Final conc. (40 µL)**
Water RNase Free	26.8	---
10X TaqMan™ Buffer	5	1X
$MgCl_2$ (25 mM)	5	2.5 mM
$dNTP_s$ (25 mM each)	0.6	300 mM
Primer HCVC1 (100 µM)	0.25	500 nM
Primer HCVC2 (100 µM)	0.25	500 nM
Probe C1 (25 µM)	0.2	100 µM
Probe C2 (25 µM)	0.2	100 µM
RNase Inhibitor (20 U/µL)	0.5	10 U
MU/V RT (50 U/µL)	0.5	25 U

Amplitaq Gold (5 U/μL)	0.5	2.5 U

TaqMan is the trademark of Roch Molecular Systems, Inc.

Table VI

Cycle	Temperature	Time	Repeat	Ramp Time	Auto Increment
Hold	48:00	60:00		Auto	
Hold	95:00	10:00		Auto	
Cycle	93:00 57:00 72:00	0:15 0.30 0.30	40	Auto	

What is claimed is:

1. A method for monitoring nucleic acid amplification comprising:

performing nucleic acid amplification on a target polynucleotide wherein the amplification is carried out using any method using a first oligonucleotide probe and a second shorter oligonucleotide probe varying in length by at least about 2 base pairs;

the first probe having a fluorophore;

the second being complementary with the first probe and having a quencher molecule capable of quenching the fluorescence of said fluorophore, the fluorophore and quencher being attached on their respective probes at positions which results in the quencher molecule quenching the fluorescence of the fluorophore when the probes are hybridized,

wherein the longer probe binds preferentially to the target polynucleotide and when preferentially bound to the target polynucleotide the fluorescence intensity of the fluorophore is greater than the fluorescence intensity of the fluorophore when hybridized to the second probe, and

monitoring the fluorescence of the flurorphore, the generation of fluorescence corresponding to the occurrence of nucleic acid amplification.

2. The method of claim 1 wherein the nucleic acid polymerase is a thermostable nucleic acid polymerase.

3. The method of claim 1 wherein the fluorophore on the first probe and the quencher molecule on the second probe are on the same hybridized base pair.

5. The method of claim 1 wherein the fluorophore and quencher molecules are within about 1 to 3 hybridized base pairs of each other.

6. The method of claim 1 wherein the flurorphore and quencher molecules are within 3 or more hybridized base pairs of each other.

7. The method of claim 1 wherein the fluorophore is on the 5' terminal nucleotide of the first probe and the quencher is on the 3' terminal nucleotide of the second probe.

8. The method of claim 1 wherein the fluorophore is on the 3' terminal nucleotide of the first probe and the quencher is on the 5' terminal nucleotide of the second probe.

9. The method of claim 1 wherein the second probe is shorter than the first probe by deletion of 3 or 3' terminal nucleotides from the nucleotide sequence of the first probe.

10. The method of claim 1 wherein the second probe is shorter than the first probe by deletion of 3 or more 3' terminal nucleotides from the nucleotide sequence of the first probe.

11. The method of claim 1 wherein the second probe is shorter than the first probe by deletion of 3 or more 5' terminal nucleotides, and deletion of 1 or more 3' terminal nucleotides of the first probe.

12. The method of claim 1 wherein the first and second probes have a disassociation temperature difference of 2 degrees or more.

13. A method for detecting the presence of specific nucleic acid sequences in a prepared nucleic acid sample comprising:

placing a sample of nucleic acids in a suitable solution and incubating with a first oligonucleotide probe and a second shorter oligonucleotide probe varying in length by about at least 2 base pairs;

the first probe having a fluorophore;

the second being complementary with the first probe and having a quencher molecule capable of quenching the fluorescence of said fluorophore, the fluorophore and quencher being attached on their respective probes at positions which results in the quencher molecule quenching the fluorescence of the fluorophore when the probes are hybridized,

wherein the longer probe binds preferentially to the target polynucleotide and when preferentially bound to the target polynucleotide the fluorescence intensity of the fluorophore is greater than the fluorescence intensity of the fluorophore when hybridized to the second probe, and

monitoring the fluorescence of the flurorphore, the generation of fluorescence corresponding to the presence of specific nucleic acid sequences.

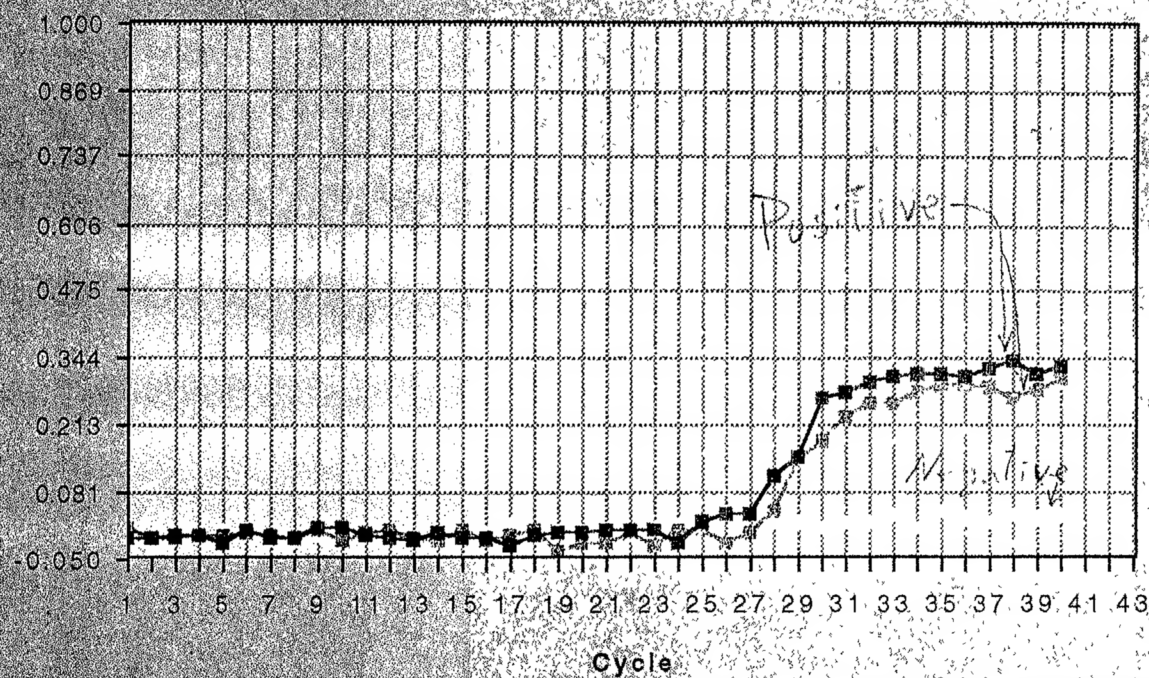
Table I

CV 03.SEQ

N		61	71	81	91	1	11	21	31
3151	SGGDIYHSVS	HARPRWFWFC	LLLLAAGVGI	YLLPNRBASE	CNTACGTRIG	INGCCAGCCC	CCTGATGGGG	GCGACACTCC	ACCATGAATC
N		51	61	71	81	91	1	11	21
3241	ACTCCCCTGT	GAGGAACTAC	TGTCTTCACG	CAGAAAGCGT	CTAGCCATGG	CGTTAGTATG	AGTGTCGTGC	AGCCTCCAGG	ACCCCCCCTC 329(
						Primer F →			
N		41	51	61	71	81	91	1	11
3331	F CCGGGAGAGC	CATAGTGGTC T	TGCGGAACCG	GTGAGTACAC	CGGAATTGCC	AGGACGACCG	GGTCCTTTCT	TGGATAAACC	CGCTCAATGC
	Probes (C1, C2)								
N		31	41	51	61	71	81	91	1
3421	CTGGAGATTT	GGGCGTGCCC	CCGCAAGACT	GCTAGCCGAG	TAGTGTTGGG	TCGCGAAAGG	CCTTGTGGTA	CTGCCTGATA	GGGTGCTTGC
N		21	31	41	51	61	71	81	91
3511	GAGTGCCCCG	GGAGGTCTCG	TAGACCGTGC	ACCATGAGCA	CGAATCCTAA	ACCTCAAAGA	AAAACCAAAC	GTAACACCAA	CCGTCGCCCA
			← Primer R						

Abstract

A method is provided for detecting the presence of nucleotides or monitoring nucleotide amplification. It utilizes fluorescence energy transfer by competitive hybridization. Competitive hybridization is achieved by using unequal length complementary probes which have a fluorophore on one probe and a quencher on the other. The fluorophore and quencher are juxtaposed in a manner wherein the proximity of the quencher to the fluorophore produces quenching of the fluorescence of the fluorphore .



Samples

- ☒ FAM - B1
- ☒ FAM - B2
- ☒ FAM - B3
- ☐ FAM - B4

Viewer: ΔRn (B...

Reporter: FAM

Threshold Cycle Calculation

Method: Threshold ...

Threshold

Use Threshold: 1.266

Mult. * Stddev: 10.0 .127

Omit Threshold: 2.0

Recommend

Baseline

Start: 3 Stop: 15

	Threshold Cycle	Baseline Mean	Baseline Stddev
FAM - B1	40.000	0.000	0.009
FAM - B2	40.000	0.000	0.007
FAM - B3	40.000	0.000	0.008
FAM - B4	20.877	0.000	0.874

Calculate Threshold Cycle

Docket No.: CL85012

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

"FLUORESCENCE ENERGY TRANSFER BY COMPETITIVE HYBRIDIZATION"

the specification of which (check one)
[X] is attached hereto.
[ ] was filed on as Serial No.
and was amended on (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below any foreign application for patent or Inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

Number	Country	Filing Date	Priority Claimed	
			Yes	No

I hereby claim the benefit under Title 35, United States Code, Section 119(e) of any United States provisional application(s) listed below.

Application Number	Filing Date
60/039,583	February 28, 1997

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

Serial No.	Filing Date	Status

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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